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Polymorphisms in the human paraoxonase (*PON1*) promoter

Victoria H. Brophy[c], Michele D. Hastings[a], James B. Clendenning[b], Rebecca J. Richter[a], Gail P. Jarvik[c,d] and Clement E. Furlong[a,c]

The University of Washington Departments of [a]Genetics, [b]Biotechnology and Medicine, Divisions of [c]Medical Genetics and [d]Epidemiology

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Paraoxonase (PON1) is a protein component of high-density lipoprotein (HDL) particles that protects against oxidative damage to both low-density lipoprotein and HDL and detoxifies organophosphorus pesticides and nerve agents. A wide range of expression levels of PON1 among individuals has been observed. We examined the promoter region of PON1 for genetic factors that might affect PON1 activity levels. We conducted a deletion analysis of the *PON1* promoter region in transient transfection assays and found that cell-type specific promoter elements for liver and kidney are present in the first 200 bp upstream of the coding sequence. Sequence analysis of DNA from a BAC clone and a YAC clone identified five polymorphisms in the first 1000 bases upstream of the coding region at positions −108, −126, −162, −832 and −909. Additionally, the promoter sequences of two individuals expressing high levels of PON1 and two individuals expressing low levels of PON1 were analysed. The two polymorphisms at −126 and −832 had no apparent effect on expression level in the reporter gene assay. The polymorphisms at position −909, −162 (a potential NF-I transcription factor binding site) and −108 (a potential SP1 binding site) each have approximately a two-fold effect on expression level. The expression level effects of the three polymorphisms appear not to be strictly additive and may depend on context effects.

Keywords: PON1, promoter polymorphism, atherosclerosis, gene expression, organophosphorus insectisides

Introduction

Paraoxonase (PON1) is tightly associated with high-density lipoprotein (HDL) particles. Recent evidence shows that PON1 protects both low-density lipoprotein and HDL from oxidation (Mackness *et al.*, 1991, 1993; Watson *et al.*, 1995; Mackness *et al.*, 1997; Aviram *et al.*, 1998; Mackness *et al.*, 1998a), a major step in the progression of atherosclerosis and heart disease. PON1 also protects against cholinesterase inhibition by the bioactive forms of organophosphorus pesticides such as diazinon (Li *et al.*, 2000) and chlorpyrifos (Li *et al.*, 1993; Li *et al.*, 1995; Shih *et al.*, 1996; Li *et al.*, 2000). PON1 also hydrolyses the nerve agents soman and sarin (Davies *et al.*, 1996). Individuals show large variation in the expression levels of PON1 (La Du *et al.*, 1986; Furlong *et al.*, 1989; Davies *et al.*, 1996; Richter & Furlong, 1999).

Two polymorphisms have been identified in the coding sequence of human PON1, L55M and Q192R. $PON1_{L55}$ is correlated with higher PON1 activity and mRNA levels than $PON1_{M55}$ (Blatter Garin *et al.*, 1997; Leviev *et al.*, 1997; Mackness *et al.*, 1998b). However, there is considerable variation among individuals with some $PON1_{M55}$ individuals having greater PON1 levels than some $PON1_{L55}$ individuals (Brophy *et al.*, 2000). The position 192 polymorphism results in substrate-dependent differences in the kinetics of hydrolysis of various substrates (Davies *et al.*, 1996; Li *et al.*, 2000). $PON1_{Q192}$ has a higher V_{max} for diazoxon and sarin hydrolysis than $PON1_{R192}$ whereas the opposite is true for the substrate paraoxon (Li *et al.*, 2000). Additionally, within each genotype, there is a great deal of variation in PON1 expression and activity level.

Correspondence to Clement E. Furlong, Department of Genetics, Box 357360, University of Washington, Seattle, WA 98195–7360, USA
Tel: +1 206 543 1193; fax: +1 206 543 0754; e-mail:clem@u.washington.edu

While the L55M polymorphism is associated with this variability, it is not likely to be the source.

Experiments on the relationship between PON1 levels and resistance to the oxon forms of diazinon and chlorpyrifos have clearly demonstrated that high PON1 levels are protective while low PON1 levels result in sensitivity. *PON1* knockout mice (missing both plasma and liver PON1) are five- to 10-fold more sensitive to cholinesterase inhibition by diazoxon or chlorpyrifos oxon than wild-type mice (Shih *et al.*, 1996; Furlong *et al.*, 1998; Li *et al.*, 2000). Reconstitution of the plasma PON1 in *PON1* null mice by intraperitoneal injection of human PON1 reconstitutes resistance to diazoxon and chlorpyrifos oxon (Li *et al.*, 2000). Our recent studies on the relationship between PON1 status (genotype and phenotype) and carotid artery disease have shown that PON1 levels are lower in patients homozygous for $PON1_{Q192}$ or heterozygotes compared to matched controls (Jarvik *et al.*, 2000).

Since variation in PON1 levels has been shown to be physiologically significant, we examined the 5′ regulatory region for genetic factors responsible for the large observed inter-individual differences in levels. Recently, two groups (Leviev & James, 2000; Suehiro *et al.*, 2000) reported on their analyses of the *PON1* promoter region. Leviev and James found three polymorphisms at −107, −824 and −907 relative to the start codon. Suehiro *et al.* found two additional polymorphisms at −126 and −160 and confirmed the one at −107 (−108 in their report). Two of the polymorphisms, −107/108 and −824, were found to have effects on PON1 expression levels. We independently identified the five polymorphisms and report additional data on the effect of the polymorphisms on PON1 expression.

Materials and methods

DNA sources

PON1 promoter sequences came from three sources: a BAC clone (GS1-155M11, Genbank accession no. AC004022), a YAC clone (A97E9; Green *et al.*, 1995; Clendenning *et al.*, 1996), and DNA from volunteers who were participants in an Epidemiology Research and Information Center project at the Puget Sound Veterans Affairs Health Care System (PSVAHCS). The study was approved by both the University of Washington and the PSVAHCS human subject review processes. Subjects gave their written informed consent. Subject DNA was prepared from buffy coat preparations by a modification of the procedure of Miller *et al.* (1988) using Puregene reagents (Gentra, Minneapolis, MN, USA). The YAC DNA was prepared by a standard method (Hoffman & Winston, 1987).

Cloning

Regions 5′ to the *PON1* gene were cloned by polymerase chain reaction (PCR). A 5.3 kb region was amplified from the YAC clone using the Extend PCR kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and the primers: CCGTAGGTACCCCAGAG GATTCTGTAATAAC and GTAATCTCGAGTCGGGGA TAGACAAAG. The PCR product was first cloned using the Topo-XL kit (Invitrogen, Carlsbad, CA, USA), then cloned into pGL3-Basic (Promega, Madison, WI, USA) using the restriction sites *Kpn*I and *Xho*I. The mammalian expression vector pGL3-Basic contains a multiple cloning site upstream of a firefly luciferase gene that has no promoter. A 6.2 kb region was amplified from the YAC clone using the TaqPlus Long kit (Stratagene, La Jolla, CA, USA), TOPO-XL cloned, then transferred 5′ to the 5.3 kb promoter to generate an 11.5 kb region upstream of the PON1 ATG (primers CTATGGTACCGTTGTACCCGTGA CAGTT and GCAGCCATGGAATACTTTAATGTTCC). A 960 bp region was amplified from the YAC clone using Taq polymerase (Promega) and the primers CCGTAACGCGTGAAGGAAAGAGACATGGAGC and GTAATCTCGAGTCGGGGATAGACAAAG. The product was Topo-TA (Invitrogen) cloned and transferred to pGL3-Basic using *Xho*I and *Mlu*I. The 200 bp fragment was generated by *Sac*I digestion of the 960 bp fragment and the other fragments were generated by *Exonuclease*III digestion (Life Technologies/Gibco BRL, Rockville, MD, USA) of the 5.3 kb region by a standard protocol (Beckler, 1996). Promoter regions of 960 bp were amplified from the volunteers and cloned as described above. The haplotypes discussed here are listed in Table 1. The QuikChange (Stratagene) site-directed mutagenesis system was employed to generate the BAC haplotype from haplotype C1/C2 for testing in the transient transfection system. Additionally, haplotypes E and G were generated by site-directed mutagenesis from the YAC haplotype while haplotype F was generated from the BAC haplotype. Cycle sequencing was used to verify that only the intended changes had been introduced.

Sequencing

The PON1 5′ regions were sequenced after cloning using Amplitaq FS or BigDye (PE Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequence analysis and alignment used the online Biology Workbench software (NCSA, http://biology.ncsa.uiuc.edu/) and CLUSTALW (Higgins *et al.*, 1992; Thompson *et al.*, 1994). Clones were sequenced at least twice to ensure quality of the

Table 1. PON1 Promoter Haplotypes

Position	*BAC*	*YAC*	*A*[a]		*B*[a]		*C*[a]		*D*[a]		*E*[b]	*F*[b]	*G*[b]
			1	*2*	*1*	*2*	*1*	*2*	*1*	*2*			
−108	C	T	T	C	C	C	C	C	C	C	C	C	T
−126	C	G	G	G	G	G	G	G	G	G	G	C	G
−162	A	G	G	G	G	G	A	A	A	G	G	A	A
−832	A	G	G	G	G	A	A	A	G	G	G	A	G
−909	G	C	C	G	G	G	G	G	G	G	C	C	C
L55M	L	M	L/M[c]	L/M[c]	L	L	L	L	L	L			
Q192R	Q	Q	Q	Q	R	R	Q	Q	R	R			
PON1 level	NA	NA	Low PON1		Low PON1		High PON1		High PON1		NA	NA	NA

[a]Haplotypes cloned from volunteers; 1 and 2 represent the two haplotypes cloned from that individual. [b]Haplotype generated by site-directed mutagenesis. [c]Heterozygote, phase with promoter was not determined. NA, not applicable.

data. The sequences of the regions subcloned from the YAC clone agreed with the published sequence (BAC GS1-155M11, Genbank accession no. AC004022) except at five positions: −C108T, −G126C, −G162A, −G832A, and −C909G where the base immediately preceding the ATG is −1. These five polymorphisms were found in two separate subclones of the YAC clone, confirming that they were not errors introduced by PCR. For the haplotypes generated from the volunteers, genotypes were verified by identifying multiple clones with the same haplotype and sequencing the PCR products directly.

Transient transfection

The pGL3 plasmids containing the various PON1 promoter regions were transiently transfected into the human kidney cell line 293 (Graham *et al.*, 1977) and the human hepatoma cell line HepG2 (Knowles *et al.*, 1980). Transfection into 293 cells used 10 µl of lipofectin (Life Technologies) and 0.5 pmol of plasmid DNA. Transfection into HepG2 cells used 15 µl of lipofectin and 0.5 pmol of plasmid. Control plasmid pRL/CMV (50 ng) containing the Renilla luciferase driven by the CMV promoter was cotransfected with the promoter/reporter construct being tested. After 24 h, the lipofectin and DNA were removed by culture medium replacement and the cells were allowed to grow an additional 24 h in normal media before being lysed and analysed using the Dual-Luciferase Assay System (Promega). Data were normalized for transfection efficiency by the Renilla luciferase activity and by total protein concentration. Luciferase activities were determined on a Turner TD-20/20 Luminometer (Sunnyvale, CA, USA). Determinations of *P*-values were determined by *t*-test.

PON1 status determination

$PON1_{192}$ and $PON1_{55}$ genotypes were determined using PCR techniques and *Alw*I and *Nla*III restriction enzyme analysis (Humbert *et al.*, 1993). PON1 paraoxon and diazoxon hydrolysis rates were measured spectrophotometrically with lithium heparin plasma, as described (Davies *et al.*, 1996; Richter & Furlong, 1999). A two-dimensional plot of diazoxon hydrolysis activities versus paraoxon hydrolysis activities provided individual PON1 phenotypes and an accurate confirmation of $PON1_{192}$ genotype.

Results

We began our analysis by asking where the important promoter elements are in the region 5′ to the *PON1* gene. Computer analysis (Schug & Overton, 1997) of the sequence immediately upstream of the

gene revealed that *PON1* contains neither a canonical TATA nor a CAAT box and the region is GC-rich, typical of TATA-less promoters. We used deletion analysis to locate important regions in the *PON1* upstream sequence. A YAC clone (A97E9) was identified that contained the region 5′ to the *PON1* coding sequence (Clendenning *et al.*, 1996). Using PCR and standard cloning techniques, plasmids were generated containing 200, 960, 5294 bp, or 11.5 kb of sequence directly 5′ to the *PON1* gene. These sequences were cloned into pGL3-Basic (Promega), 5′ to the reporter gene firefly luciferase. Additional plasmids were generated by *Exonuclease*III digestion from the 5′ end of the 5294 bp fragment, providing plasmids with promoter regions of varying lengths.

Studies in mice (Primo-Parmo *et al.*, 1996) and rabbits (Hassett *et al.*, 1991) have demonstrated that PON1 is most highly expressed in the liver and HepG2 human liver cells have previously been shown to express PON1 (Navab *et al.*, 1997; Feingold *et al.*, 1998). In mouse kidney, PON1 is expressed at approximately half the level that occurs in liver (Primo-Parmo *et al.*, 1996). Thus, 293 human kidney cells were employed to identify the location of cell-type specific elements. Each plasmid was cotransfected into the liver HepG2 cells with the control plasmid pRL-CMV (Promega). A subset of the plasmids was similarly transfected into the 293 kidney cell line. Figure 1 shows the various lengths of the constructs and their relative activities in the transient transfection system, standardized to the pGL3-Basic plasmid with no promoter.

In the HepG2 cells, the smallest fragment tested, 200 bp upstream of the *PON1* gene, showed considerable promoter activity. Increasing the length of the promoter region had little effect except that a slight downward trend in expression level was observed with increasing lengths. The 5 kb region produced only 35% luciferase activity compared to the 960 bp region ($P = 0.04$). The 11.5 kb region, however, produced somewhat more activity than the 5 kb fragment. Four of the promoters were tested for activity in the 293 cells, resulting in a similar pattern of expression (Fig. 1). The level of luciferase activity, however, was lower in the 293 kidney cells than the HepG2 liver cells.

A fragment almost 1 kb in length was chosen for sequence analysis. The published sequence from BAC GS1-155M11 was compared to the sequence we obtained from the YAC clone (A97E9), both generated from human genomic libraries. We identified five polymorphisms between the BAC and the YAC sequences at positions −108, −126, −162, −832 and −909, where the A of the ATG start codon is +1

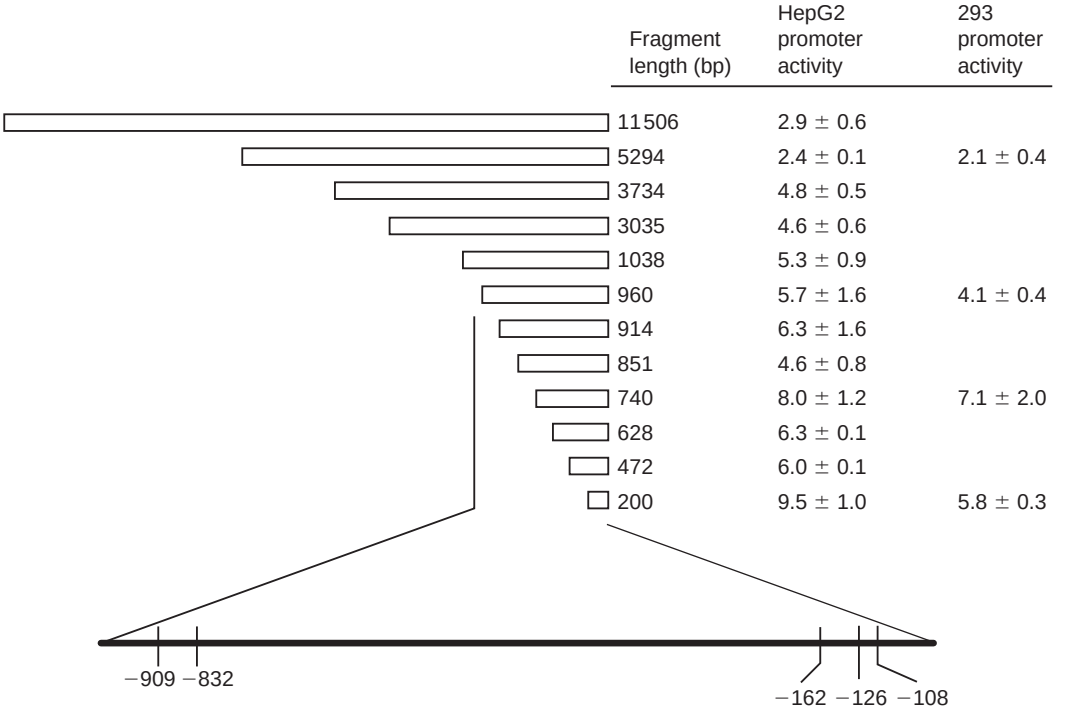

Fig. 1. The lengths of the promoter regions examined and the relative luciferase activity generated by each promoter in the HepG2 cells and selected promoters in 293 cells. The luciferase activity from the plasmid with no promoter was set as 1 and the data presented as fold activation over background. The locations of the polymorphisms are shown.

and the base immediately preceding it −1. We also sequenced the PON1 promoter regions from four individuals. We chose two individuals with very low PON1 activity levels and two with very high levels. We did not find any additional polymorphisms but the sequences did contain various combinations (haplotypes) of the polymorphisms at −108, −162, −832 and −909. The only instance of the −126C was in the BAC sequence (Table 1).

To analyse the functional consequences of the polymorphisms, the published BAC haplotype and three additional haplotypes were generated by site-directed mutagenesis and the various natural and generated haplotypes were subjected to transient transfection in HepG2 cells (Fig. 2). The haplotype found in the YAC clone produced a relatively low expression level while the G haplotype produced the highest expression level and the other haplotypes produced a range of intermediate expression levels. Comparison of the various haplotypes allowed determination of the effect of each polymorphism on expression level (Table 2). The polymorphisms at −126 and −832 had no statistically significant effect on expression level. The polymorphisms at −108, −162 and −909 each affected relative luciferase expression by approximately two-fold.

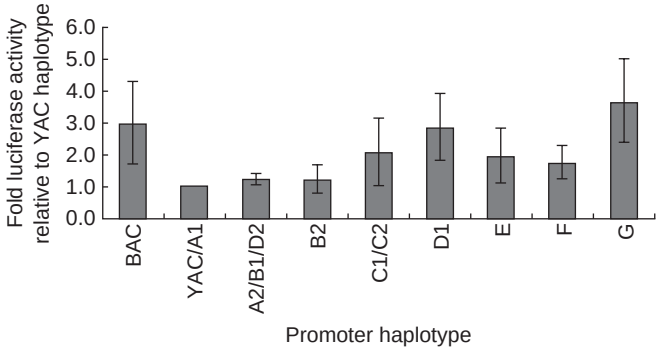

Fig. 2. Fold induction of luciferase activity for the various PON1 promoter haplotypes. The luciferase activity generated from the plasmid containing the YAC PON1 promoter sequence was set as 1. Table 1 shows the details for each haplotype.

Discussion

In this study using cultured human cells, as observed previously in mouse (Primo-Parmo *et al.*, 1996) and rabbit (Hassett *et al.*, 1991) organs, we found that liver cells express more PON1 than kidney cells. The 293 kidney cells show the same pattern of expression levels as the HepG2 liver cells, but the degree of activation is lower relative to the background established by the promoterless plasmid. The data revealed that the promoter elements responsible for this cell-type-specific expression pattern are present in the first 200 bp upstream of the PON1 coding sequence. The CMV promoter driving the cotransfected *renilla* gene on the pRL-CMV control plasmid is strong and constitutively activated and has the potential to transactivate the test plasmid (Farr & Roman, 1992). The amount of control plasmid was kept low and the cell-type-specific expression pattern of the PON1 promoter fragments support the conclusion that

Table 2. Analysis of polymorphisms' functional effects by comparison of haplotypes differing at only one position

Polymorphism	*Haplotype*[a]	*Expression Level*[b]	P *value*[c]
−108	*T*GGGC (YAC/A1)	1.0 ± 0	< 0.001
	*C*GGGC (E)	1.9 ± 1.0	
−126	CCAAG (BAC)	3.0 ± 1.3	0.164
	CGAAG (C1/C2)	2.1 ± 1.1	
−162	CGGGG (A2/B1/D2)	1.2 ± 0.2	0.003
	CG*A*GG (D1)	2.8 ± 1.1	
	CGGAG (B2)	1.2 ± 0.5	0.050
	CG*A*AG (C1/C2)	2.1 ± 1.1	
	TGGGC (YAC/A1)	1.0 ± 0	< 0.001
	TG*A*GC (G)	3.6 ± 1.4	
−832	CGA*A*G (C1/C2)	2.1 ± 1.1	0.176
	CGAGG (D1)	2.8 ± 1.1	
	CGGG*G* (A2/B1/D2)	1.2 ± 0.2	0.991
	CGG*A*G (B2)	1.2 ± 0.5	
−909	CCAA*G* (BAC)	3.0 ± 1.3	0.040
	CCAA*C* (F)	1.7 ± 0.6	

[a]Polymorphism order, from left to right: −108, −126, −162, −832, −909; haplotype name in parentheses (see Table 1 for details; polymorphism that differs in italics). [b]Luciferase activity, fold induction relative to the YAC haplotype. [c]*t*-test.

transactivation is not occurring, and that the firefly luciferase expression is a direct result of the PON1 promoter sequence.

As the length of *PON1* promoter sequence increased, the reporter gene activity decreased, most notably with the 5 kb region which produced a luciferase activity level that was only 2.4-fold over background (compared to 9.5-fold for the 200 bp region). There may be a negative regulatory element(s) present that accounts for the reduction in activity. Counteracting sequences may be present elsewhere, such as in an intron. Since the 960 bp fragment produced good expression and contained the five polymorphisms, this fragment was chosen for further analysis.

Recently, Leviev and James (2000) published an analysis of polymorphisms they identified in the *PON1* promoter (Genbank accession no. AF051133). They found three polymorphisms at −107, −824 and −907 that correspond to the polymorphisms described here at −108, −832 and −909, respectively. Additionally, Suehiro *et al.* (2000) identified polymorphisms at −108, −126 and −160, the last of which likely corresponds to the −162 polymorphism identified in this work. Base counting and sequence differences among the laboratories most likely account for the discrepancies. The sequences we obtained from the YAC clone and the individuals correspond to the sequence published for BAC GS1-155M11.

The polymorphisms were analysed for functional significance by comparison of haplotypes that differed at only one position, either using naturally occurring haplotypes isolated from the individuals or haplotypes generated by site-directed mutagenesis (Table 2). We did not detect a change in the reporter gene expression level for the −126 polymorphism. We did not find an effect by the −832 polymorphism either, although Leviev and James found that the −832 polymorphism produced a two-fold change in expression level (Leviev & James, 2000). Additionally, we detected an approximately two-fold change in expression level for the −909 polymorphism whereas Leviev and James found no effect. The discrepancies may be a consequence of interactions among the polymorphisms resulting in context effects. The exact haplotypes tested by Leviev and James were not listed in their publication, precluding comparison with the haplotypes described here.

The −T108C polymorphism resulted in a 1.9-fold change in expression level. The MatInspector program (Quandt *et al.*, 1995), using the TRANSFAC database (Heinemeyer *et al.*, 1999) identified a potential binding site for SP1, a ubiquitous activation factor, at position −108. The data described here are in agreement with Leviev & James (2000) and Suehiro *et al.* (2000).

The −162 polymorphism identified in this work resulted in a 1.8–3.6-fold change in expression level, depending on the haplotype context. The MatInspector program identified a potential NF-I transcription factor binding site when an A is present at −162, consistent with the observation that the higher expressing constructs are driven by −162A containing alleles. NF-I, also known as CTF, is a ubiquitous nuclear factor and a transcriptional activator (Nagata *et al.*, 1982; Gronostajski *et al.*, 1988; Santoro *et al.*, 1988).

The data presented here support approximately two-fold effects for the −108, −162 and −909 polymorphisms individually. When the three polymorphisms are examined together, however, the effect does not appear to be additive. For example, the haplotype YAC/A1 has the lower activity version of all three polymorphisms while D1 has the higher activity versions. One would expect a six-fold difference in expression level yet the data showed only 2.8-fold activation. Additionally, the G haplotype has the highest expression level but only one of the three high activity polymorphisms (−162A). These results lend further support for context effects and interactions among the factors that bind in the vicinity of the polymorphisms.

Individual D is a heterozygote for the *PON1* promoter region. One allele yields high expression in the transient transfection assay while the other yields low expression, yet the serum from this individual showed very high levels of PON1. These data support the hypothesis that *PON1* DNA sequence may not be the sole determinant of PON1 expression levels. For example, cigarette smoke (Nishio & Watanabe, 1997) and smoking (James *et al.*, 2000) have been shown to inhibit PON1. Thus, environment, diet (Shih *et al.*, 1996, 1998) and interactions with other gene products (Mackness *et al.*, 1987, 1989) are likely to have an effect on PON1 serum levels.

PON1 activity level variation among individuals is at least 13-fold (Furlong *et al.*, 1989; Davies *et al.*, 1996) and low levels of PON1 expression have been found to be associated with carotid artery disease (Jarvik *et al.*, 2000). The presence of leucine at amino acid 55 is associated with higher mRNA and PON1 level on average (Blatter Garin *et al.*, 1997; Leviev *et al.*, 1997), although a given individual may have a PON1 expression level that is the opposite of that expected based on $PON1_{55}$ genotype (Brophy *et al.*, 2000). As suggested previously (Leviev & James, 2000), the $PON1_{55}$ effect may be due to linkage to promoter polymorphisms rather than a direct effect by the coding region genotype. The previous reports

found that the *PON1* promoter polymorphisms at −107/−108 and −824/−832 contribute to PON1 expression level. Here we have provided evidence that the −162 and −907/−909 polymorphisms also contribute to PON1 activity level and thus may contribute to an individual's risk for heart disease and susceptibility to specific organophosphorus toxins. Interestingly, polymorphisms in the promoter of the HIV coreceptor gene CCR5 have been shown to affect disease progression in AIDS (Martin *et al*., 1998; Clegg *et al*., 2000), suggesting promoter polymorphisms may modulate the severity of many diseases.

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